

## Genetics and Physiology of Proline Utilization in *Saccharomyces cerevisiae*: Enzyme Induction by Proline

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Proline is converted to glutamate in the yeast *Saccharomyces cerevisiae* by the sequential action of two enzymes, proline oxidase and  $\Delta^1$ -pyrroline-5-carboxylate (P5C) dehydrogenase. The levels of these enzymes appear to be controlled by the amount of proline in the cell. The capacity to transport proline is greatest when the cell is grown on poor nitrogen sources, such as proline or urea. Mutants have been isolated which can no longer utilize proline as the sole source of nitrogen. Mutants in *put1* are deficient in proline oxidase, and those in *put2* lack P5C dehydrogenase. The *put1* and *put2* mutations are recessive, segregate 2:2 in tetrads, and appear to be unlinked to one another. Proline induces both proline oxidase and P5C dehydrogenase. The arginine-degradative pathway intersects the proline-degradative pathway at P5C. The P5C formed from the breakdown of arginine or ornithine can induce both proline-degradative enzymes by virtue of its conversion to proline.

Intersecting metabolic pathways sharing intermediates often have unique regulatory properties to aid the cell in maintaining physiological balance. The pathway for the degradation of proline to glutamate in the yeast *Saccharomyces cerevisiae* has intermediates in common with both the proline biosynthetic pathway and the arginine-ornithine catabolic sequence. Ideally, the cell should avoid degrading proline needed for protein synthesis and prevent unnecessary expression of the catabolic enzymes in the absence of the appropriate substrate.

*S. cerevisiae* cannot use proline as the sole source of carbon and nitrogen or as the sole source of carbon, but it can use proline as the sole source of nitrogen. The pathway of this utilization is the same as that described for *Escherichia coli* (8), *Salmonella typhimurium* (6, 14), *Aspergillus nidulans* (1), and mitochondrial preparations from the livers and kidneys of mammals (17, 18). Proline is converted to  $\Delta^1$ -pyrroline-5-carboxylate (P5C) by proline oxidase. P5C dehydrogenase acts on the P5C to form glutamate.

This paper describes the proline-degradative pathway in the wild-type yeast and characterizes proline-nonutilizing mutants. We also determined the inducer of the pathway to be proline. An accompanying paper (5) describes the isolation and characterization of a mutant strain which has high levels of the proline-degradative enzymes in the absence of inducer.

### MATERIALS AND METHODS

**Yeast strains.** The strains of *S. cerevisiae* employed in this study are listed in Table 1. They are all isogenic apart from the specified genotype.

**Media.** The minimal medium used was yeast nitrogen base without ammonium sulfate and amino acids (Difco Laboratories, Detroit, Mich.) to which appropriate supplements were added.

For growth in liquid or on plates, the minimal medium was supplemented with 0.5% galactose as the sole source of carbon and any one of the following as the source of nitrogen: ammonium sulfate (0.2%), glutamate (monosodium salt, 0.1%), proline (0.1%), urea (0.1%), arginine hydrochloride (0.1%), or ornithine hydrochloride (0.1%).

For tetrad analysis, the minimal plates were supplemented with histidine (20 mg/liter) when appropriate. Yeast extract-peptone-dextrose plates contained 2% glucose, 1% yeast extract (Difco), 2% peptone (Difco), and 2% agar (Difco). Sporulation medium contained 0.3% potassium acetate.

**Mutagenesis.** Mutations were induced by treatment with ethyl methane sulfonate according to the method of Fink (7). The mutagenized cultures were cultured in minimal medium containing galactose and glutamate or in yeast extract-peptone-dextrose medium.

**Isolation of proline-nonutilizing mutants.** Mutagenized cells of the wild-type strain MB1000 were spread on agar plates containing galactose and glutamate and were incubated at 30°C for 3 to 5 days. The colonies were replica plated to galactose-proline and galactose-glutamate plates and incubated at 30°C for 2 days. Colonies which failed to grow on the galactose-proline plates but grew on the galactose-glutamate

TABLE 1. *Strains used*

Strain	Description	Source
MB1000	$\alpha$ Wild type	$\Sigma$ 1278b of J.-M. Wiame
MB1057	<b>a</b> <i>his4-42</i>	
MB1122	$\alpha$ <i>put1-54</i>	
MB1123	$\alpha$ <i>put2-57</i>	
MB1135	$\alpha$ <i>pro1-59</i>	
MB1136	$\alpha$ <i>pro2-60</i>	
MB1142	$\alpha$ <i>pro3-66</i>	
MB209-3A	<b>a</b> <i>his4-42 put1-54</i>	
MB281-10C	$\alpha$ <i>put2-57 pro3-66</i>	
MB282-2D	<b>a</b> <i>put2-57 pro1-59</i>	
MB283-1C	<b>a</b> <i>put2-57 pro2-60</i>	

medium were purified by subcloning on the permissive medium.

Galactose was used as the sole carbon source to select against respiratory-deficient yeast strains which are (trivial) proline nonutilizers due to their lack of mitochondrial function (13).

**Genetic analysis.** Haploid strains of opposite mating types were allowed to mate on yeast extract-peptone-dextrose medium at 30°C overnight. The resulting diploids were isolated by selection for prototrophy and were purified by subcloning on selective medium. To induce sporulation, diploid strains grown in liquid yeast extract-peptone-dextrose medium were transferred to sporulation medium and incubated at 30°C for 2 days. Asci were dissected by micromanipulation after digestion with Glusulase (Endo Laboratories, Garden City, N.Y.).

**Chemicals and substrates.** *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) buffer, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, and DL-plus-allo- $\delta$ -hydroxylysine hydrochloride were purchased from Calbiochem, La Jolla, Calif. NAD and *o*-aminobenzaldehyde were purchased from Sigma Chemical Co., St. Louis, Mo.

DL- $\Delta^1$ -Pyrroline-5-carboxylate was synthesized by periodate oxidation of DL- $\delta$ -hydroxylysine as described by Williams and Frank (19). For use in enzyme assays, the P5C was neutralized with 10 N potassium hydroxide to a final pH of 7.0 immediately before use.

Uniformly labeled L-[<sup>14</sup>C]proline was obtained from New England Nuclear Corp., Boston, Mass.

**Growth of cells for enzyme assays.** Cells grown for subsequent P5C dehydrogenase assays were treated as described previously for the P5C reductase determination (4). For proline permease and proline oxidase assays, 10- to 15-ml amounts of the appropriately supplemented minimal medium in 125-ml side arm flasks were inoculated with a stationary-phase inoculum to a density of 2 to 10 Klett units (blue filter) by using a Klett-Summerson colorimeter. Cultures were incubated at 30°C with shaking until their densities reached approximately 100 Klett units.

**Preparation of cell extracts for the P5C dehydrogenase assay.** The procedure used was identical to that described previously for the P5C reductase assay (4).

**Enzyme assays. (i) Proline permease.** This assay was used to determine the capacity for proline uptake at the time of cell harvest. A 5-ml amount of

culture was removed and filtered through a membrane filter (0.45  $\mu$ m; Millipore Corp., Bedford, Mass.). The cells collected on the filter were washed with 2 20-ml volumes of minimal medium lacking carbon and nitrogen sources and kept at room temperature. The filter containing the cells was then placed in 5 ml of pre-warmed, pre-aerated minimal medium containing 2% glucose and no nitrogen source in a flask shaking at 30°C for 5 min. Cycloheximide was then added (final concentration, 1  $\mu$ g/ml) to inhibit protein synthesis. After 5 min, labeled proline (0.5 mCi/mmol) was added to a final concentration of 0.2 mM, and 0.5-ml samples were removed every 20 s for 2 min 20 s and filtered by using 0.45- $\mu$ m Millipore filters. Each sample was washed three times with 15 ml of minimal medium containing 0.1% unlabeled proline kept at room temperature. The filters were dried under heat lamps, placed in 4 ml of scintillation fluid (Scinti-Verse; Fisher Scientific Co., Fair Lawn, N.J.), and counted in a Beckman LS-250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). During the time course, essentially no radioactivity was found in protein, as determined by cold trichloroacetic acid precipitation. The assay was linear with time up to 5 min and with cell concentration, and the slope of the curve of counts per minute versus time was used as the initial velocity of uptake. Each value given is the average of at least two, and usually more, determinations.

**(ii) Proline oxidase (EC 1.4.3.2).** Cells from 2-ml samples of the culture were collected by filtration on Millipore filters and immediately immersed in liquid nitrogen for 10 s. Each filter was then placed in a small test tube containing 0.5 ml 0.1 M HEPES buffer (pH 7.5) with 3 mM MgCl<sub>2</sub> and kept on ice. When all of the filters from one culture had been prepared, they were assayed immediately. Each tube was blended in a Vortex mixer vigorously to transfer the cells from the filter to the buffer and placed at room temperature. A 0.4-ml portion of 10% proline was added to each tube, which was blended in a Vortex mixer vigorously for 10 s and incubated without shaking at 30°C. At 4 min, 0.1 ml of *o*-aminobenzaldehyde (6 mg/ml in 20% ethanol) was added, followed by 0.5 ml of 10% trichloroacetic acid to stop the reaction. The tubes were then blended in a Vortex mixer again, and color was allowed to develop for 30 min. The filters were removed, and the debris was spun out. The absorbance was read at 443 nm. The millimolar extinction coefficient of the P5C-*o*-aminobenzaldehyde complex is 2.71 (17). Each value given represents the average of at least two, and usually more, determinations.

Zero-time blanks were run by adding the proline, *o*-aminobenzaldehyde, and trichloroacetic acid rapidly in succession and blending in a Vortex mixer. This value was always subtracted from the 4-min value for the net activity and was identical to assay blanks in which water was substituted for proline with incubation at 30°C for 4 min.

The yeast proline oxidase activity is very unstable. Cultures cannot be allowed to stand at room temperature or on ice, intact or rendered permeable by liquid nitrogen, without loss of activity.

When the growth conditions described are used, the

proline oxidase assay is linear both with time (up to 10 min) and with cell concentration.

(iii) **P5C dehydrogenase (EC 1.5.1.12).** The assays were carried out at 30°C by using a Zeiss recording spectrophotometer. The assay mixture contained, in 1 ml: 0.05 M TAPS buffer (pH 9.0), 1.5 mM NAD, 5 mM P5C (pH 7.0), and 10 to 20  $\mu$ l of extract. Reactions were initiated by the addition of substrate. Enzymatic activity was monitored by following the increase in absorbance at 340 nm. The background value obtained by omitting the extract was subtracted from each assay. This value was approximately 0.003 units of absorbance at 340 nm per min. The formation of NADH was linear with time (up to 10 min) and with cell concentration. Each value given represents the average of at least two, and usually more, determinations.

**Protein determinations.** Protein was determined by the method of Lowry et al. (10), using bovine serum albumin as a standard. A modification of this procedure was used on whole cells (16).

## RESULTS

**Enzyme levels.** The results shown in Table 2 indicate that the proline uptake system is fully expressed on poor nitrogen sources (urea, proline) but does not require proline as an inducer and is repressed by ammonia. When the cells were grown on a combination of urea and proline, the level of uptake was only about one-third of the maximal level.

Proline oxidase and P5C dehydrogenase are both subject to induction by proline; the levels of these enzymes were very low in cells grown in the absence of proline and were highest when proline served as the sole nitrogen source. In medium containing both ammonia and proline, both enzymes were intermediate in specific activity. This result probably reflects the ammonia

TABLE 2. Specific activities of enzymes of proline catabolism in the wild-type strain MB1000

Nitrogen source <sup>a</sup>	Sp act of:		
	Proline permease <sup>b</sup>	Proline oxidase <sup>c</sup>	P5C dehydrogenase <sup>d</sup>
NH <sub>3</sub>	1.5	1.4	11.6
Urea	22.9	1.9	ND <sup>e</sup>
NH <sub>3</sub> + proline	2.5	7.5	23.8
Urea + proline	7.6	18.5	ND
Proline	23.2	26.6	96.9

<sup>a</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was supplied at 0.2% and urea and proline were supplied at 0.1%. The carbon source was 0.5% galactose.

<sup>b</sup> Expressed as initial velocity (nanomoles of [<sup>14</sup>C]-proline taken up per minute per milligram of protein).

<sup>c</sup> Expressed as nanomoles of P5C formed per minute per milligram of protein.

<sup>d</sup> Expressed as nanomoles of NADH formed per minute per milligram of protein.

<sup>e</sup> ND, Not determined.

repression of the proline permease and the resulting low internal concentration of proline.

The permease and the two enzymes showed fairly large induction ratios for a eucaryotic organism. The permease and oxidase increased 15-fold in specific activity, and the dehydrogenase increased 9- to 10-fold.

The lowest levels of proline oxidase specific activity varied from 0 to 2. Therefore, small differences of the uninduced level are not significant.

**Isolation and genetic analysis of proline-nonutilizing mutants.** Proline-nonutilizing mutants were isolated as described above. These were colonies growing on plates containing glutamate as the sole nitrogen source which failed to grow when replica plated to proline-containing plates. The genetic symbol *put* (for proline utilization) is proposed for these mutants.

The initial isolates were crossed to strain MB1057 (a *his4-42*). The *put*<sup>-</sup> mutations were recessive to wild type and segregated 2 *put*<sup>+</sup>:2 *put*<sup>-</sup> in tetrads, as expected for mutations in single nuclear genes.

Complementation testing indicated two classes of mutations existed; these were called *put1* and *put2*.

The results of tetrad analysis of two diploid strains containing the *put1* and *put2* genes indicated no close linkage to each other, mating type, or the *his4* marker also segregating in the cross (data not shown).

**Enzyme deficiencies of the proline non-utilizers.** As Table 3 shows, *put1* mutants had reduced proline oxidase activity, whereas *put2* mutants were deficient in P5C dehydrogenase

TABLE 3. Specific activities of proline-degradative enzymes in proline-nonutilizing mutants<sup>a</sup>

Mutant	Nitrogen source	Sp act of:		
		Proline permease <sup>b</sup>	Proline oxidase	P5C dehydrogenase
<i>put1-54</i> <sup>c</sup>	NH <sub>3</sub>	1.8	0.5	11.9
	NH <sub>3</sub> + proline	5.3	0.2	68.9
	Urea + proline	6.2	0.2	ND <sup>d</sup>
<i>put2-57</i> <sup>c</sup>	NH <sub>3</sub>	1.6	2.0	3.7
	NH <sub>3</sub> + proline	3.5	24.0	3.6
	Urea + proline	2.2	36.3	ND

<sup>a</sup> The carbon and nitrogen sources and the specific activity units were as described in the footnotes to Table 2.

<sup>b</sup> The initial uptake velocity on urea-containing medium for both strains was comparable to that of the wild type.

<sup>c</sup> MB1122.

<sup>d</sup> ND, Not determined.

<sup>e</sup> MB1123.

activity. Since these strains could not grow in a medium containing proline as the sole nitrogen source, a second nitrogen source had to be provided to allow growth. The presence of an additional nitrogen source affected the activity of the proline transport system and the level of internal proline, as discussed above.

Strains containing the *put1* mutation are notable in another respect; their P5C dehydrogenase activity was found to be three times the wild-type specific activity on ammonia-proline medium (68.9 versus 23.8); it was the same as the wild-type activity on a medium containing ammonia alone. In analogous fashion, *put2* strains had three times the level of proline oxidase compared with the wild type (specific activity, 24 versus 7.5), approaching the fully expressed level of the wild type when grown on proline as the sole nitrogen source.

When the cells were grown on a medium containing urea and proline, the proline oxidase level of the *put2* mutants was even higher than the fully induced level found in the wild-type strain grown on proline as the sole nitrogen source.

In the *put1* strain, the initial velocity of the proline permease of cells grown on ammonia-proline medium was approximately two fold higher than the initial velocity in the wild-type strain. The *put2* strain also had slightly higher initial uptake velocities for proline, although the effect was smaller. It is not clear that these differences, although reproducible, are significant.

**Identification of the inducer of proline oxidase.** The mutant lacking P5C dehydrogenase (*put2*) appeared to be more sensitive to proline as an inducer of proline oxidase than the wild-type strain was; when grown on a medium containing ammonia as well as proline, it contained a level of proline oxidase three times higher than that of the wild-type strain, which corresponded to the fully induced enzyme level of the wild type growing on proline alone. This suggested the possibility that P5C, which could not be further degraded in the *put2* mutant, would accumulate and serve as a potent inducer of the proline oxidase. Alternatively, the P5C might be converted back to proline by the action of P5C reductase, the last enzyme in the proline biosynthetic pathway (4).

To identify the inducer of proline oxidase, strains were constructed carrying two mutations; one was in *put2*, and the other was in the proline biosynthetic pathway, either early in the pathway, *pro1* or *pro2*, affecting a step before P5C, or *pro3*, eliminating P5C reductase. Figure 1 indicates the locations of these mutations. Table 4 gives the results of the proline oxidase assays

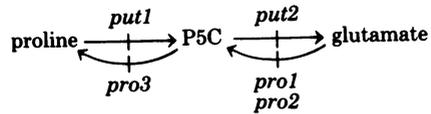


FIG. 1. Pathways of proline metabolism. Mutations: *put1*, proline oxidase; *put2*, P5C dehydrogenase; *pro1* and *pro2*, unspecified lesions early in proline biosynthesis; *pro3*, P5C reductase.

TABLE 4. Determination of the inducer of proline oxidase

Strain	Genotype	Proline oxidase sp act on NH <sub>3</sub> + proline <sup>a</sup>
MB1000	$\alpha$ Wild type	7.5
MB1123	$\alpha$ <i>put2-57</i>	24.0
MB1135	$\alpha$ <i>pro1-59</i>	3.6
MB1136	$\alpha$ <i>pro2-60</i>	3.3
MB1142	$\alpha$ <i>pro3-66</i>	7.5
MB282-2D	<b>a</b> <i>put2-57 pro1-59</i>	27.2
MB283-1C	<b>a</b> <i>put2-57 pro2-60</i>	17.4
MB281-10C	<b>a</b> <i>put2-57 pro3-66</i>	6.9

<sup>a</sup> Galactose (0.5%) was the carbon source; results are expressed as nanomoles of P5C formed per minute per milligram of protein.

on cells of these strains grown on a medium containing ammonia and proline. In strains MB282-2D and MB283-1C with an intact P5C reductase, the proline oxidase levels are high, just as in the *put2* strain. In strain MB281-10C, in which, because of the absence of the reductase, P5C cannot be converted to proline, the level of proline oxidase has the same low value as in the wild-type or the *pro3* strain. This result strongly suggests that proline made from the P5C which accumulates in the *put2* mutant is the inducer of proline oxidase. In the absence of a functioning P5C reductase, as in strain MB281-10C, P5C cannot induce proline oxidase to a higher activity than the activity found in the wild-type strain growing under the same conditions.

We have no explanation for the finding that the *pro1* and *pro2* strains had consistently lower proline oxidase activities than the *pro3* strain.

Arginine and ornithine should both induce the proline-degradative enzymes in strains containing an intact P5C reductase since both compounds can be degraded to P5C and subsequently converted to proline. Proline oxidase levels of specific activity of the wild type were 17.4 and 18.8 when the cells had been grown on arginine and ornithine, respectively, as the sole nitrogen source. These values were lower than when proline served as the sole nitrogen source but equal to the value found on urea-proline

medium and 10-fold higher than the uninduced level.

**Determination of the inducer of P5C dehydrogenase.** It was observed (Table 3) that a proline oxidase-deficient (*put1*) strain had three times the P5C dehydrogenase activity found in the wild type when grown on a medium containing ammonia and proline. Since the *put1* strain cannot metabolize the proline to P5C, we conclude that the internally accumulating proline induces the P5C dehydrogenase.

## DISCUSSION

The pathway of proline degradation in yeast is highly regulated. The proline permease was reported to be specific and different from the general amino acid permease (*gap*), which is also highly ammonia sensitive (9). Grenson et al. (9) reported that proline uptake was identical in a wild-type strain and a *gap* mutant and that proline did not inhibit the uptake of other amino acids by the general amino acid permease. They concluded that proline was not a substrate of the general amino acid permease.

The proline permease appears to control the level of expression of the catabolic enzymes by determining the intracellular level of the inducer, proline. The level of the proline permease appears to be determined by the type of nitrogen source rather than by the amino acid (proline) itself. If the transport system for proline is inducible at all, this response is a minor component of its regulation. The highest proline uptake measured occurred when the cells were grown on either proline alone or urea alone, both poor nitrogen sources as measured in terms of growth rate. These results agree with the description of a starvation-induced proline permease in *Saccharomyces chevalieri* which was reported by Schwencke and Magana-Schwencke (15).

Apparently, the sensing device of the cell which determines the level of proline uptake does not monitor the ability of the cell to use those nitrogen sources. One might expect the proline uptake capability of a *put1* mutant growing on urea-proline medium to be as high as the wild-type level on urea-containing medium since it cannot use the proline provided. This is, however, not the case. Such a mutant growing on urea-proline medium has a low initial velocity of proline uptake.

The regulation of proline degradation in the yeast cell seems to be organized in such a way that only one component of the system, the proline permease, needs to be sensitive to the presence of nitrogen sources preferred over proline. The proline oxidase and P5C dehydrogenase respond to proline and not to the presence of ammonia.

The yeast system differs in this respect from the proline-degradative pathway in *Aspergillus*. In *A. nidulans*, under comparable conditions of low catabolite repression, the major proline permease is not repressible by ammonia, and the two catabolic enzymes are (1).

A P5C dehydrogenase-deficient mutant in yeast was reported previously by Lundgren et al. (11). It was derived from a glutamate auxotroph which was blocked in the tricarboxylic acid cycle and had become unable to use proline to supply the auxotrophic requirement for glutamate.

The degradation of arginine or ornithine in yeast also results in the production of the intermediate P5C. When either compound is used as the sole nitrogen source, the proline oxidase levels are elevated, suggesting that some of the P5C is converted to proline by the P5C reductase. The cell must have P5C dehydrogenase activity in order to complete the metabolism of arginine from ornithine to glutamate. Whether there is also an arginine-inducible P5C dehydrogenase in addition to the proline-inducible one is not currently known.

Both Bechet and Wiame (3) and Middelhoven (12) reported the presence of an inducible P5C dehydrogenase in their studies of arginine catabolism, but neither determined its inducer. Middelhoven (12) found arginase and ornithine transaminase were both induced by arginine and, less efficiently, by ornithine. By using the *put2* (P5C dehydrogenase-deficient) mutant, we are now in a position to study an arginine-specific P5C dehydrogenase, if one exists.

Although genes of related function are rarely clustered in the eucaryotic systems which have been studied in detail, the genes for proline degradation in *Aspergillus*, unlike those in yeast, are closely linked. The *Aspergillus* proline permease and P5C dehydrogenase genes are separated from the proline oxidase gene and a positive regulatory element by an intervening regulatory region (2). In the bacterium *S. typhimurium* all of the elements of this system are typically clustered, although one gene seems to code for the structure of both proline oxidase and P5C dehydrogenase (14).

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